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Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

93117452.8

PRIORITY DOCUMENT

Der Präsident des Europäischen Patentamts:
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

Den Haag, den
The Hague,
La Haye, le 23/11/94

Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

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2

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Adeno-Associated Virus - its Diagnostic Use with Early Abortion

The present invention relates to a method of detecting a causative agent of the so-called spontaneous early abortion by investigating patients' samples for the presence of adeno-associated virus DNA (AAV DNA), or AAV antigen or antibodies, preferably of the IgM type, directed to AAV.

The adeno-associated viruses (AAV) which are human parvoviruses that depend on coinfecting helper viruses for their replication, are thought to be non-pathogenic (Siegl, G. et al. (1985), Intervirology, 23, pp. 61-73; Berns, K. I. et al. (1987), Adv. Virus Res., 32, pp. 243-306) but rather to exhibit tumorsuppressive properties (Rommelaere et al. (1991) J. Virol. Methods, 33, pp. 233-251). The virus may persist in infected individuals, possibly by integration of its DNA into specific chromosomal sites of the host cell genome as seen in cell culture. Recent studies of our laboratories have demonstrated that AAV is able to induce differentiation in a variety of cells of human and mouse origin (Klein-Bauernschmitt et al. (1992) J. Virol., 66, pp. 4191-4200) including embryonic stem cells. In the course of looking for putative targets of AAV infection, we analyzed material from spontaneous abortion for the presence of AAV DNA using for example the polymerase chain reaction (PCR), the Southern blotting technique and the in situ hybridization technique. Additionally, we analyzed serum samples from women with miscarriage and from other diseased or healthy women for the presence of antibodies to AAV using serological standard techniques such as enzyme linked immunsorbent assay (ELISA),

fluorescenceimmuno assay (FIA), radioimmuno assay (RIA) or immunofluorescence assay (IFA).

Surprisingly, we found a significant correlation of both detectable AAV DNA in samples of abortion material and detectable IgM antibodies directed to AAV with the early abortion occurring during the first trimester of pregnancy.

Disclosure of the invention:

Accordingly, the present invention relates to a method of detecting the causative agent of spontaneous abortion comprising the steps of

(a) hybridizing a probe for an AAV polynucleotide to nucleic acids of a sample of abortion material under conditions which allow the formation of a heteroduplex between an AAV nucleic acid and the probe, and

(b) detecting a polynucleotide duplex which contains the probe.

In a preferred embodiment of the invention the method of detecting the causative agent of spontaneous abortion is a polymerase chain reaction (PCR), Southern blotting or in situ hybridization technique.

In another preferred embodiment of the invention a hybridization technique is applied as described above, wherein one or more nucleic acid probes are used which are selected from the group consisting of the primers pan1, pan3, nest1 and nest 2. In figure 1 a schematic drawing of these primers, relative to the genome of the AAV type 2 (AAV-2) and the nucleotide sequences of the primers is presented.

The present invention further relates to a method of detecting the causative agent of spontaneous abortion comprising the steps of

(a) incubating a probe antibody directed against an AAV antigen with a sample of abortion material under conditions which allow the formation of an antigen antibody complex, and

(b) detecting the antigen antibody complex containing the probe antigen

In a preferred embodiment of the present invention the method of antigen detection as described above is an enzyme linked immunosorbent assay (ELISA), a radioimmuno assay (RIA), a fluorescence immuno assay (FIA) or an immunofluorescence assay (IFA).

Furthermore, the present invention relates to a method of detecting the causative agent of spontaneous abortion comprising the steps of

(a) incubating a sample containing AAV or an antigenic part thereof with a sample suspected of containing anti-AAV antibodies under conditions which allow the formation of an antibody-antigen complex, preferably only containing antibodies of the IgM type, and

(b) detecting an IgM antibody-antigen complex containing the probe antigen.

In another preferred embodiment of the present invention the method of detection of AAV specific IgM antibodies is an ELISA, a RIA, a FIA or an IFA.

The present invention further relates to a kit for detecting the causative agent of spontaneous abortion by hybridization as described above, comprising a probe for an AAV polynucleotide in a suitable container.

The present invention further relates to a kit for detecting the causative agent of spontaneous abortion by immunological antigen detection as described above, comprising a probe antibody directed against an AAV antigen in a suitable container.

The present invention further relates to a kit for detecting the causative agent of spontaneous abortion by immunological antibody detection as described above, comprising AAV or an antigenic part thereof in a suitable container.

Modes for carrying out the invention:

The art is rich in methods available to the man of the art in recombinant nucleic acid technology, microbiology and immunobiology for carrying out the present invention. Detailed descriptions of all of these techniques will be found in the relevant literature. See for example: Maniatis, Fitsch & Sambrook: Molecular Cloning: A Laboratory Manual (1989); DNA Cloning, Vol. I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed. 1984); Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds., 1984); Animal Cell Culture (R. I. Freshney ed., 1986); J. D. Watson, M. Gilman, J. Witkowski, M. Zoller: Recombinant DNA, Second Edition (1992); Immunochemical Methods in Cell and Molecular Biology (Academic Press, London, 1987); Protein Purification: Principles and Practice, Second Edition (Springer Verlag, N.Y.); Handbook of Experimental Immunology, Vol. I-IV (D. M. Weir and C. C. Blackwell eds., 1986); Immunoassay: A Practical Guide (D. W. Chan and M. T. Perlstein eds., 1987); ELISA and Other Solid Phase Immunoassays: Theoretical and

Practical Aspects (D. M. Kemeny and S. J. Challacombe eds., 1988); Principles and Practice of Immunoassay (C. P. Price and D. J. Newman eds., 1991).

More detailed information on specific methodological aspects of AAV, such as cell culture, virus growth, virus purification, isolation of proteins, preparation of specific antibodies directed to AAV can be found in the relevant literature, e.g. in Ruffing, M. et al. (1992), J. Virol., 66, pp. 6922-6930 and Rittner, K. et al. (1992), J. Gen. Virol., 73, pp. 2977-2981.

All reagents such as antigens, antibodies, probe antigens, probe antibodies, nucleic acid probes, primers and auxilliary reagents necessary to perform an immunoassay or a hybridisation assay, possibly using amplification techniques for improved sensitivity may be filled into suitable containers or coated to any solid phase such as plastic, glass and cells, and packaged into kits together with instructions for conducting the test.

Example 1:

Detection by Polymerase-Chain-Reaction (PCR) - analysis of AAV DNA in biological, e.g., curettage material of spontaneous abortion.

The primers used in PCR (pan1, pan3) and nested PCR (nest1, nest2), respectively, were designed to hybridize to sequences of AAV-2 and AAV-5 DNA by allowing mismatches not leading to amplification of other (e.g., cellular) DNA sequences. The amplified products are distinguishable by Southern blot experiments. The primers were prepared according to standard procedures.

The primers were designed displaying mismatches (underlined) as shown below:

	------AACTGGACCAATGAAAACTTTCC-------	pan1
1386	TGCGTAAACTGGACCAATGAGAACTTTCCCTTCAAC	AAV-2
130	TGCGTAAACTGGACCAATGAAAACTTTCCCTTCAAC	AAV-5

	AAAAAGTCTTTGACTTCCTGCTT	pan3
1729	AAAAAGTCTTTGACTTCCTGCTT	AAV-2
472	AAAAAGTCCTTGACTTCCTGCTT	AAV-5

DNA prepared from histological sections (5 μm, of fresh or fixed, paraffin-embedded, deparaffinated material [Methods as described by D. H. Wright and M. M. Manos in "PCR Protocols, A Guide to Methods and Applications", edited by M. A. Innis, D. H. Gelfand,

J. J. Snoisky and T. L. White, Chapter 19, pp. 153-158; Academic Press, New York, 1990] were analysed by PCR using the primers pan1 and pan3 combined, followed in AAV-positive cases by a repetition of the PCR (to confirm specificity) using the (internal) primers nest1 and nest2 (see figure), respectively. PCRs were performed for 40 cycles (one cycle = 92°C, 1 min; 62°C, 4 min; 92°C, 15 sec) (van den Brule et al. (1989) J. Med. Virol., 29, pp. 20-27). Amplified products were characterized by electrophoretic separation (2 % agarose gel) and blotting onto a nylon membrane (Gene Screen, NEN, Dupont, Dreieich, Germany) followed by hybridization at high stringency with ^{32}P-labelled probes (labelled using the Megaprime ^{TM} DNA Labelling System, Amersham, UK) of AAV-2 (pTAV2 [Heilbronn et al. (1990), J. Virol., 64, pp. 3012-3018]) or of AAV-5. This probe was cloned from DNA from purified AAV-5 virions, propagated with adenovirus type 12 and purified as described in de La Maza and Carter (1980), J. Virol., 33, pp. 1129-1137 and in Rose (1974) Parvovirus Reproduction, pp. 1-61; In: H. Fraenkel-Conrat and R. R. Wagner, eds., Comprehensive Virology, Plenum Press, New York.

Table 1

388 bp part of BamH1b fragment of AAV5

487	TCAATCAGGTGCCGGTGACTCACGAGTTTAAAGTTCCCAGGGAATTGGCGGGAACTAAAG AGTTAGTCCACGGCCACTGAGTGCTCAAATTTCAAGGGTCCCTTAACCGCCCTTGATTTC	546
547	GGGCGGAGAAATCTCTAAAACGCCCACTGGGTGACGTCACCAATACTAGCTATAAAAGTC CCCGCCTCTTTAGAGATTTTGCGGGTGACCCACTGCAGTGGTTATGATCGATATTTTCAG	606
607	TGGAGAAGCGGGCCAGGCTCTCATTTGTTCCCGAGACGCCTCGCAGTTCAGACGTGACTG ACCTCTTCGCCCGGTCCGAGAGTAAACAAGGGCTCTGCGGAGCGTCAAGTCTGCACTGAC	666
667	TTGATCCCGCTCCTCTGCGACCGCTCAATTGGAATTCAAGGTATGATTGCAAATGTGACT AACTAGGGCGAGGAGACGCTGGCGAGTTAACCTTAAGTTCCATACTAACGTTTACACTGA	726
727	ATCATGCTCAATTTGACAACATTTCTAACAAATGTGATGAATGTGAATATTTGAATCGGG TAGTACGAGTTAAACTGTTGTAAAGATTGTTTACACTACTTACACTTATAAACTTAGCCC	786
787	GCAAAAATGGATGTATCTGTCACAATGTAACTCACTGTCAAATTTGTCATGGGATTCCCC CGTTTTTACCTACATAGACAGTGTTACATTGAGTGACAGTTTAAACAGTACCCTAAGGGG	846
847	CCTGGGAAAAGGAAAACTTGTCAGATTT GGACCCTTTTCCTTTTGAACAGTCTAAA	874

Example 2:

Detection by Southern blot analysis of AAV DNA in fresh curettage material

Genomic DNA was isolated using standard procedures with minor modification (Laird et al. (1991), Nucl. Acids Res., Vol. 19, pp. 4293-4294) and digested with restriction enzymes allowing analysis of characteristic restriction sites within the AAV genome. After separation through 0.8 % agarose gels, DNA fragments were blotted onto Nylon membranes (Gene Screen) and hybridized AAV-2 DNA (pTAV2, see example 1) or specific AAV-5 DNA (see table 1) labelled by random priming with [α-^{32}P] dCTP (Amersham, Braunschweig, Germany).

Example 3:

Detection of AAV DNA by in situ hybridization in sections of biopsy material, e.g., curettage from spontaneous abortion

In situ hybridisation was performed as described (Tobiasch et al. (1992) Differentiation, 50, pp. 163-178), however, with the modification that AAV-2 DNA was detected by RNA-DNA hybridization. After DNase treatment, the probes were subjected to limited alkaline hydrolysis. Upon linearisation of the plasmid pTAV2 (Heilbronn et al. (1990), see above) with EcoRV, riboprobes were obtained and labelled with [^{35}S]-UTP by in vitro transcription with T7 RNA polymerase (method as described in Boehringer Mannheim Prodedure supplied with the "SP6/17 Transcription Kit"). Prior to hybridization, both probe and target DNA were denatured (93°C, 10 min). For in situ hybridization with [^{32}P-]-UTP labelled probes, the protocol was as described in Dürst et al. (1992) Virology, 189, pp. 132-140.

Example 4:

ELISA for the detection of IgG antibodies directed to AAV

96-well microtiterplates (Nunc, Denmark) were coated with 50 µl CsCl-gradient purified AAV 2 (dilution 1:1000 in 0.05 M carbonate-buffer pH 9.6) or with 50 µl recombinant AAV 2 capsid proteins VP1-3 (1:8000 in 0.05 M carbonate-buffer) and incubated over night at RT. Plates were washed twice (washing buffer: PBS, 0,05 % Tween 20) and human sera were added (50 µl/well, dilutions 1:25 to 1:800, dilution buffer: PBS, 2 % BSA, 0.05 % Tween 20) and incubated for 1 h at 37°C in a wet chamber. After washing plates were incubated with 50 µl/well peroxidase conjugated donkey anti-human IgG antibody (1:2000) for 45 minutes at 37°C in a wet chamber. Plates were washed four times and 50 µl substrate solution (5 mg OPD in 25 ml 0.1 M citratebuffer pH 5.0 + 10 µl H_2O_2 35 %) was added. Plates were stored for 10-15 minutes in the dark and the reaction was stopped with 50 µl 1N H_2SO_4/well. Extinctions were measured at 492 nm in a Titertek photometer. Background signal was determined by measuring the extinction without adding human sera and was substracted on every well (background signal extinction ranged from 0.035 to 0.05).

Example 5:

ELISA for the detection of IgM antibodies directed to AAV

Plates were coated as described in example 4. Human sera were added after they had been treated according to the following absorption protocol in order to eliminate remaining IgG-antibodies : 20 µl absorption reagent (FREKAFluor, Fresenius, Germany) were diluted with 25 µl PBS and 5 µl of human serum was added. Absorption was performed for at least 15 minutes at RT, and subsequently sera were tested at dilutions from 1:100 to 1:800. Incubation was performed for 1 h at 37°C in a wet chamber and after washing 50 µl/well peroxydase conjugated goat anti human IgM antibody (1:2000 in PBS/2 % BSA/0.05 % Tween 20) were added. Plates were incubated for 45 minutes at 37°C and washed four times. The OPD reaction and photometric evaluation were performed as described in example 4.

Example 6:

Detection of AAV-DNA in curettage material of spontaneous abortion

A total of 50 samples of curettage material of spontaneous abortion were analysed for the presence of AAV DNA either by PCR or Southern Blot or both. 41 samples were from abortions in the first and 9 samples from abortions in the second and third trimester of pregnancy.

Among the 41 samples taken during the first trimester of preganancy, 14 consisted of fresh material that could be tested by Southern Blot, by which method 9 samples were shown to be positive. All other samples tested were sections from paraffin-embedded tissues, that were analysed by PCR. Among these, 30 samples were from abortions in the first trimester of pregnancy, of which 12 samples were shown to be positive for AAV DNA. All of the 9 samples from the second or third trimester of pregancy were negative by PCR.

Thus, in 21 of 41 samples, i.e. 50 % of spontaneous abortions in the first trimester of pregnancy AAV specific DNA sequences could be detected, whereas 9 spontaneous abortions in the second or third trimester were negative (see table 2).

Table 2

Prevalence of AAV DNA in curettage material

Diagnosis / Pathology	Detection of AAV DNA by (number AAV positive / number analyzed)		
	PCR	Southern Blot	Total
spontaneous abortion (1st trimester of pregnancy)	12/30	9/14	21/41*
abortion 2nd trimester	0/3	n.d.	0/3
abortion 3rd trimester or placenta post partum	0/6	n.d.	0/6

n.d. = not done;
* = 3 samples positive with PCR were tested by Southern blot analysis

Example 7:

A total of 148 serum samples drawn from healthy probands, diseased patients with various syndromes being unrelated to abortion, and pregnant women with spontaneous abortion during the first trimester of pregnancy were tested for antibodies directed to AAV.

The results obtained are displayed in table 3. Generally, the prevalence of specific IgG antibodies was quite high, between 62 and 100 % in the different groups of probands/patients. However, specific IgM antibodies were shown to be significantly correlated with "pregnancy problems".

Tab. 3

Serum Antibodies to AAV Diagnosis	n	IgG- IgM-	IgG+ IgM-	IgG- IgM+	IgG+ IgM+	IgG+ n	%	IgM+ n	%
Controls (all)	***58***	***8***	***45***	***2***	***3***	***48***	***83***	***5***	***8,6***
Employees	32	4	24	2	2	26	81	4	12,5
Patients*)	26	4	21	0	1	22	85	1	4
breast (all)	***38***	***1***	***32***	***0***	***5***	***37***	***97***	***5***	***13,2***
mammary dystrophy	19	1	13	0	5	18	75	5	26
breast cancer	19	0	19	0	0	19	100	0	0
cervix uteri (all)	***26***	***2***	***17***	***4***	***3***	***20***	***77***	***7***	***27***
normal (or metaplasia)	3	1	2	0	0	2	67	0	0
CIN / CIS	22	1	14	4	3	17	77	7	32
cancer	1	0	1	0	0	1	100	0	0
pregnancy problems (all)	***26***	***6***	***12***	***2***	***6***	***18***	***69***	***8***	***31***
Extra uterine	2	0	2	0	0	2	100	0	0
chromosomal aberrations	3	0	2	0	1	3	100	1	33
abortion (1st trimester) of unclear etiology	21	6	8	2	5	13	62	8	38

*) with uterus myoma, or normal pregnancy, hysterectomy (normal)

Claims:

1. A method of detecting the causative agent of spontaneous abortion comprising the steps of

   (a) hybridizing a probe for an AAV polynucleotide to nucleic acids of a sample of abortion material under conditions which allow the formation of a heteroduplex between an AAV nucleic acid and the probe, and

   (b) detecting a polynucleotide duplex which contains the probe.

2. The method as claimed in claim 1, which is a PCR, Southern blotting or an in situ hybridization technique.

3. The method as claimed in claim 1, wherein one or more probes are used which are selected from the group consisting of the primers pan1, pan3, nest1 and nest2.

4. A method of detecting the causative agent of spontaneous abortion comprising the steps of

   (a) incubating a probe antibody directed against an AAV antigen with a sample of abortion material under conditions which allow the formation of an antigen antibody complex, and

   (b) detecting the antigen-antibody complex containing the probe antigen.

5. The method as claimed in claim 4, which is an ELISA, a RIA, a FIA or an IFA.

6. A method of detecting the causative agent of spontaneous abortion comprising the steps of

   (a) incubating a sample containing AAV or an antigenic part thereof with a sample suspected of containing anti-AAV antibodies under conditions which allow the formation of an antibody-antigen complex, preferably only containing antibodies of the IgM type, and

   (b) detecting an IgM antibody-antigen complex containing the probe antigen.

7. The method as claimed in claim 6, which is an ELISA or an IFA.

8. A kit for performing the method as claimed in claim 1, comprising a probe for an AAV polynucleotide an a suitable container.

9. A kit for performing the method as claimed in claim 4, comprising a probe antibody directed aginst an AAV antigen in a suitable container.

10. A kit for performing the method as claimed in claim 6, comprising AAV or an antigenic part thereof in a suitable container.

4

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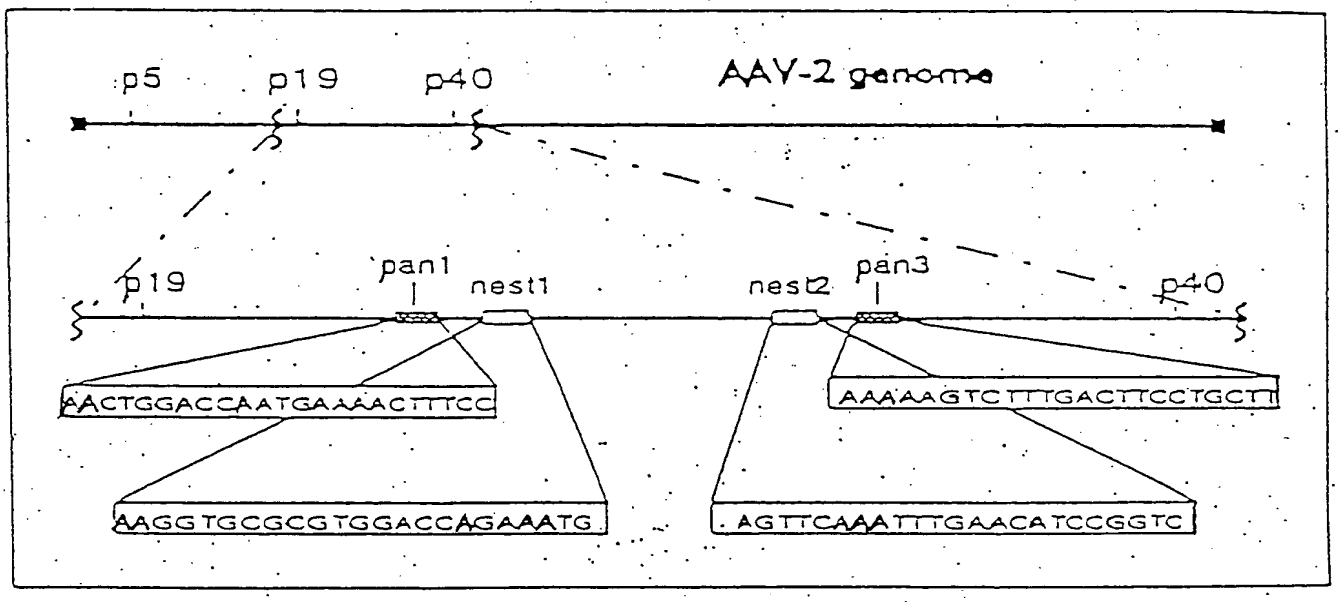
Abstract

Adeno-Associated Virus - its Diagnostic Use with Early Abortion

The present invention relates to a method of detecting a causative agent of the so-called spontaneous early abortion by investigating patients' samples for the presence of adeno-associated virus DNA (AAV DNA), or AAV antigen or antibodies, preferably of the IgM type, directed to AAV.

Figure 1


p5
p19
p40
AAV-2 genome
p19
pan1
nest1
nest2
pan3
p40
AACTGGACCAATGAAAACTTTCC
AAAAAGTCTTTGACTTCCTGCTT
AAGGTGCGCGTGGACCAGAAATG
AGTTCAAATTTGAACATCCGGTC